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Amino Acid Flow through Aphid and Its Symbiont: Studies with ^{15}N-Labeled Glutamine

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ABSTRACT—Amino acid excretion of symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum* was investigated using [ε-^{15}N]glutamine. Aphids were transferred to a synthetic diet containing [^{15}N]glutamine within 24hr after birth, and the honeydew was collected until they attained adulthood. While in the honeydew of symbiotic aphids the total content of ^{15}N remained less than 1.0 atom% excess, that in the honeydew of aposymbiotic ones gradually increased to about 6.0 atom% excess. The predominant amino acid in the former was arginine whose ^{15}N content was 0.60 atom% excess. Aposymbiotic aphids excreted great amounts of glutamine and asparagine, and the ^{15}N contents of these amino acids were 11.01 and 2.98 atom% excess, respectively. In addition to these amino acids, the honeydew of aposymbiotic aphids contained an unidentified, ninhydrin positive compound that was highly labeled with ^{15}N (6.4 atom% excess). This compound was identified as a mixture of γ-glutamylglutamine and γ-glutamylasparagine by analyses using NMR, MS and HPLC.

INTRODUCTION

Investigations of nitrogen excretion in aphids has centered on the occurrence of large amounts of amino acids in the honeydew [1, 9]. The intracellular symbionts housed in the mycetocytes play important roles in the nitrogen metabolism of aphids [6], and depletion of the symbionts changes the amino acid composition in the honeydew [13, 14]. While arginine is the most abundant amino acid in the honeydew of symbiotic aphids kept on a synthetic diet, the major amino acids in that of aposymbiotic aphids are glutamine and asparagine. Recently, we suggested that ammonia is detoxified primarily by assimilation into glutamic acid by glutamine synthetase [15]. In this study, to further investigate amino acid excretion in the endosymbiotic system of aphids, symbiotic and aposymbiotic pea aphids were fed on a synthetic diet containing [ε-^{15}N]glutamine.

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MATERIALS AND METHODS

Insects

The stock culture of aphids was maintained on young broad bean plants, *Vicia faba* (L.) at 15°C with a photoperiod of 17 hr. Aposymbiotic aphids were obtained by the rifampicin injection [7].

Tracer experiments with [ε-^{15}N]glutamine

Synthetic diet used here was that of Febvay *et al.* [4], with a small modification as described previously [14]. In tracer experiments with ^{15}N, glutamine in the diet was replaced by [ε-^{15}N]glutamine (95.0% ^{15}N, Shouko Tsusho Co.). In this diet, ^{15}N concentration in the total amino-nitrogen content was 7.6 atom% excess. Day-1 nymphs born on the plant were transferred onto this diet, and the honeydew was collected until they attained adulthood.

The ^{15}N content was measured by the emission spectrometric analysis [8]. In brief, a sample was sealed into a Pyrex glass tube with CuO and CaO

under low pressure (below 10^{-4} torr). The sample tube was then heated at 560°C for 3 hr. During this period, the sample was decomposed taking the oxygen atom from CuO and the resulting water and carbon dioxide were absorbed into CaO. After analysis in an emission spectrometer JASCO-NIA-1, ^{15}N abundance was calculated from measurement of intensity ratio of the bandheads of $^{14}N^{14}N$,$^{14}N^{15}N$ and $^{15}N^{15}N$ molecules in the nitrogen emission spectra.

To measure the total ^{15}N content in the honeydew, samples of honeydew were directly subjected to analysis. To determine the ^{15}N content in each amino acid, the honeydew was applied to an ion exchange column (Dowex ^{W}X-8, 50–100 mesh) and the amino acids retained in the column were eluted with 3N NH_4OH. The purified amino acids were then separated by two-dimensional silica gel TLC using the solvents, phenol : water (3 : 1 V/V) for the first dimension and *n*-butanol : acetic acid : water (4 : 1 : 1 v/v) for the second dimension. On staining the gel with ninhydrin reagent, the central part of each colored spot was scraped off the plate, and the amino acid was extracted with 80% ethanol into a Pyrex glass tube for analysis.

Amino acid analysis of the honeydew was carried out with reverse phase HPLC as described previously [14].

Identification of γ-glutamylamides

An unidentified ninhydrin-positive spot was found on the two dimensional TLC of the honeydew collected from aposymbiotic aphids. To identify this compound, amino acids in the honeydew were purified on an ion-exchange column as described above, and were separated by two dimensional paper chromatography using Whatman 3MM chromatography paper using first, phenol : water=3 : 1 (v/v) and then, *n*-butanol : acetic acid : water=12 : 3 : 5 (v/v). The unidentified compound was eluted from the paper with water in a moistened chamber, and then subjected to NMR, MS and HPLC analyses. ^{1}H- and ^{13}C-NMR spectra were measured on a Brucker AMX-600 spectrometer. ^{13}C-^{1}H long-range couplings were analyzed from a HMBC (Heteronuclear Multiple Bond Correlation) spectrum [2]. A negative FAB (Fast Atom Bombardment) mass spectrum was recorded on a JEOL DX-300 mass spectrometer. The reverse phase HPLC was performed as described previously [14].

Authentic γ-glutamylamides were synthesized by an enzymatic method using γ-glutamyltranspeptidase [12].

RESULTS

Tracer experiments with ^{15}N-glutamine

Aphids were transferred to a diet containing [ε-^{15}N]glutamine within 24 hr after birth, and samples of honeydew, collected until the aphids attained adulthood, were analyzed by an emission spectrometric method. In the honeydew from symbiotic aphids, the total content of ^{15}N remained less than 1.0 atom% excess for 13 days after birth (Fig. 1), suggesting that most ^{15}N molecules were retained by the aphids. Aposymbiotic aphids needed longer time for their development than symbiotic ones, and the ^{15}N content in their honeydew gradually increased to about 6.0 atom% excess.

Table 1 shows the ^{15}N contents of major amino acid constituents in the honeydew. Samples of honeydew from symbiotic and aposymbiotic aphids were collected from 10th through 13th day of birth and from 16th through 19th day of birth, respectively. One part of the sample was analyzed by HPLC, and the other was subjected to ^{15}N

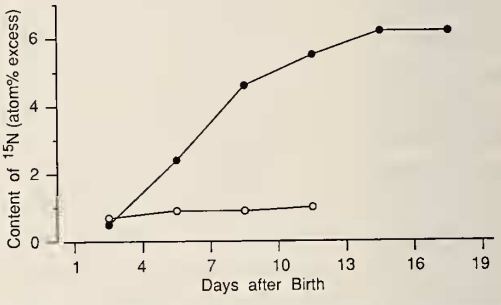

Fig. 1. Content of ^{15}N in the honeydew. Aphids were transferred onto a synthetic diet containing [ε-^{15}N]glutamine, and the samples of honeydew were collected until they attained adulthood. Values were expressed as means of two runs.
○ symbiotic aphids, ● aposymbiotic aphids.

TABLE 1. Content of ^{15}N in each amino acid in the honeydew produced by the aphids maintained on a diet containing [ε-^{15}N]glutamine

aphid	amino acid	excretion (nmol/aphid/day)	^{15}N content (atom% excess)
symbiotic	arginine	3.05	0.60
	lysine	1.06	0.20
	phenylalanine	0.99	0.39
	histidine	0.51	0.04
aposymbiotic	glutamine	3.65	11.01
	asparagine	2.03	2.98
	arginine	1.29	0.07
	lysine	1.21	0.03
	compound X	—	6.40

Values are expressed as means of two runs.

analysis after separation by two dimensional TLC. The honeydew of symbiotic aphids was rich in arginine, lysine, phenylalanine and histidine. Except histidine, these amino acids were slightly labeled, and arginine was the most highly labeled among the three.

In the honeydew of aposymbiotic aphids, glutamine, asparagine, arginine and lysine were abundant constituents. Unlike in the honeydew of symbiotic aphids, arginine and lysine were scarcely labeled with ^{15}N. By far the highest labeled amino acid was glutamine, which was probably because some [^{15}N]glutamine molecules in the diet were excreted without being utilized. In addition to glutamine, asparagine was found to be labeled. The honeydew of aposymbiotic aphids, but not symbiotic ones, contained an unidentified compound X, found as a ninhydrin positive spot on TLC, which was highly labeled with ^{15}N.

Identification of compound X

Compound X purified by two dimensional paper chromatography was subjected to NMR, MS and HPLC analyses. Although X was observed as a single spot on both TLC and paper chromatography, the ^{1}H- (Fig. 2) and ^{13}C-NMR spectra suggested that the purified sample was a mixture of two compounds in the ratio of 5:3, one consisted of glutaminyl and glutamyl residues and the other, asparaginyl and glutamyl residues. A HMBC spectrum of X gave ^{13}C-^{1}H long-range couplings between carbonyl carbons and γ-protons of glu-

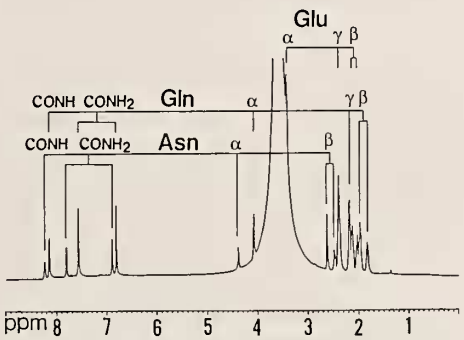

FIG. 2. A ^{1}H-NMR spectrum of compound X.

tamyl residue, suggesting that one content of the sample was γ-glutamylglutamine and the other γ-glutamylasparagine. The negative FAB mass spectrum of X gave two ion peaks at m/z 260 and 274 which were consistent with the $(M-H)^-$ ions of γ-glutamylasparagine and γ-glutamylglutamine, respectively (Fig. 3).

HPLC of compound X showed two peaks with the retention times of 7.5 min and 8.2 min (Fig. 4),

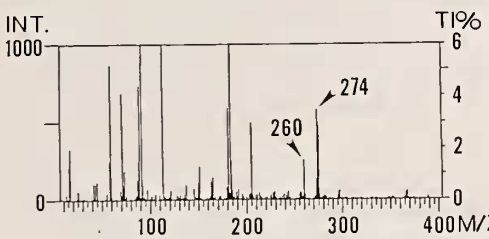

FIG. 3. A negative FAB mass spectrum of compound X.

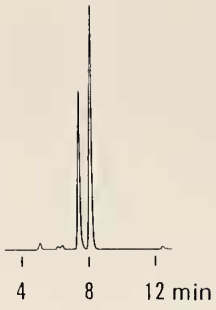

Fig. 4. Reverse phase HPLC of compound X. The sample was coupled with PITC, applied to an ODS-H column (4.6 mmϕ×25 cm) equilibrated with 0.05% triethylamine in 0.14M sodium acetate (pH 6.35): acetonitrile (47:3 V/V), and eluted by acetonitrile up to 60% at 38°C, at a flow rate of 0.8 ml/min. The effluent was monitored at 254 nm.

which accorded with those of authentic γ-glutamylasparagine and γ-glutamylglutamine, respectively. The ratio of γ-glutamylglutamine to γ-glutamylasparagine in X, calculated from the areas of the two peaks, was about 5:3, which agreed with the result observed in the ^{1}N-NMR spectrum. The excretion rate of γ-glutamylglutamine by aposymbiotic aphids was 0.8 nmol/aphid/day which corresponded to 6.7 mol% of the total amino acids excreted. Accordingly, the excretion rate of γ-glutamylasparagine can be about 0.5 nmol/aphid/day. However, its direct estimation was not feasible because the retention time of this compound was the same as that of glutamic acid.

DISCUSSION

One major finding in the present study is that aposymbiotic aphids, but not symbiotic ones, excrete γ-glutamylamides, which suggests that these are key compounds to understand amino acid metabolism in aphid. In a number of animal tissues, γ-glutamylamino acids are produced by γ-glutamyltranspeptidase in the following reaction [10]:

γ-Glutamyl-cysteinyl-glycine (glutathione) + amino acid $\rightleftarrows$ γ-glutamylamino acid + cysteinyl-glycine

Since in these tissues γ-glutamyltranspeptidase is associated with cell membrane, it has been suggested that this reaction enables free amino acids to enter the cell in the form of γ-glutamylamino acids, which later are subject to cleavage in the cytosol. As suggested in our preceding paper [15], it is likely that aphid synthesizes large amounts of glutamine and asparagine utilizing ammonia produced as a result of nitrogen metabolism. In symbiotic aphids, these two amino acids are absorbed, probably by the aid of γ-glutamyltranspeptidase, by the mycetocyte, and then converted into other nitrogenous substances including essential amino acids through metabolic pathways of intracellular symbiont. As far as this process proceeds, the γ-glutamylamides, once formed, are subject to rapid cleavage and conversion, and does not accumulate anywhere. In contrast, in aposymbiotic aphids, because of lack of conversion reactions due to symbiont, it is probable that γ-glutamylamides, as well as glutamine and asparagine, accumulate in their mycetocytes. It is conceivable that these accumulated compounds are apt to leak from the cell into the honeydew. Aside from the detailed mechanism of excretion of the γ-glutamylamides, it is no doubt that without symbiont aphids cannot make use of these compounds that they synthesize, while they have membrane-associated γ-glutamyltranspeptidase irrespective of presence or absence of symbiont (our unpublished data).

Feeding on a diet with ^{15}N concentration at 7.6 atom% excess, aposymbiotic aphids excreted honeydew containing ^{15}N at 6.0 atom% excess. Thus, they retained only a small portion of ^{15}N imbibed as glutamine from the diet. As for each amino acid in their honeydew, the only amino acid labeled, except glutamine, was asparagine. In view of its high ^{15}N content, it is likely that the amide nitrogen is transferred directly from glutamine to aspartic acid by glutamine-dependent asparagine synthetase [5].

In the honeydew of symbiotic aphids, arginine is not only the predominant constituent of amino acids, but also the most highly labeled with ^{15}N from glutamine (Table 1). This result is consistent with the earlier proposition that this nitrogen-rich amino acid, with four nitrogen atoms per mole-

cule, serves as a carrier of nitrogen waste [3, 14]. While symbiotic aphids recycle most nitrogen by way of glutamine and asparagine, they may excrete its portion mainly in the form of arginine. In fact, it has been suggested that arginine is synthesized by symbiont [11, 14].

When these results taken together with the previous ones [15], movement of amino acids through aphid and its symbiont may be summarized as shown in Fig. 5. Ammonia produced as a result of nitrogen metabolism is assimilated by glutamic acid to form glutamine, a part of which is further converted into asparagine. Most of the two amides after conversion into various nitrogenous substances by enzymes of the symbiont, are reutilized by the host. In this context, the major constituent of amino acid in the honeydew, except those amino acids that flow through the gut, is a partial fraction of arginine that is produced by symbiont, but not utilized.

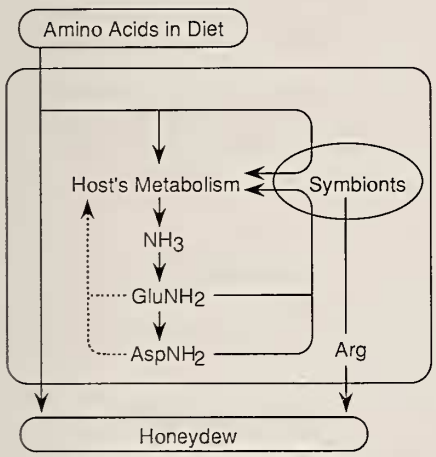

FIG. 5. A model of amino acid excretion of symbiotic aphid. See Discussion.

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